

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 88116947.8

(51) Int. Cl.⁴: **C07K 11/00 , C12P 21/04 ,
//A61K37/02,C07K9/00,
(C12P21/04,C12R1:045)**

(22) Date of filing: 13.10.88

(30) Priority: 27.11.87 GB 8727807

(43) Date of publication of application:
07.06.89 Bulletin 89/23

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(71) Applicant: **GRUPPO LEPETIT S.P.A.**
23, Via G. Murat
I-20159 Milano(IT)

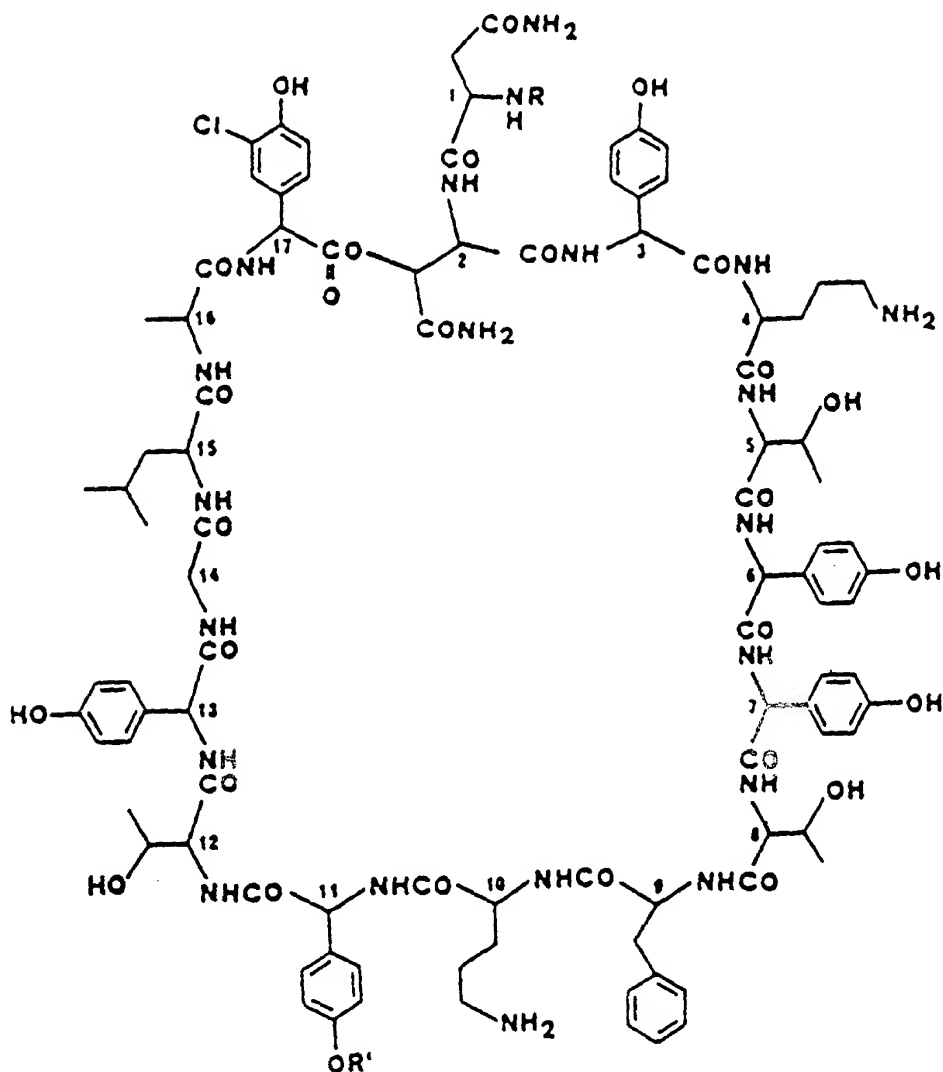
(72) Inventor: **Assi, Francesco**
34, Via Corridoni
I-20063 Cernusco Sul Naviglio (Milano)(IT)
Inventor: **Gastaldo, Luciano**
7, Via San Martino
I-20010 Pogliano Milanese (Milano)(IT)
Inventor: **Denaro, Maurizio**
41, Viale Bligny
I-20136 Milano(IT)
Inventor: **Ciabatti, Romeo**
15-A, Via Brodolini
I-20026 Novate Milanese (Milano)(IT)
Inventor: **Cavalleri, Bruno**
25, Via Pierlombardo
I-20135 Milano(IT)

(54) **Novel antibiotic compounds named A/16686 Factors A'1, A'2 and A'3.**

(57) The invention relates to depsipeptidic compounds related to antibiotic A/16696 complex. Said compounds are named antibiotic A/16686 factors A'1, A'2 and A'3, respectively and are produced by fermentation of the microorganism Actinoplanes sp ATCC 33076 or a natural or artificial mutant thereof under appropriate conditions or by contacting antibiotic A/16686 factors A1, A2 and A3 or a mixture thereof with the mycelium of Actinoplanes sp ATCC 33076 or a natural or artificial mutant thereof. The compounds have antibacterial activity, in particular, against widely diffused gram-positive bacteria and are particularly useful for topical treating of wound infections and acne.

EP 0 318 680 A2

5
10
15
20
25
30
35
40



45

50

and the acid addition salts thereof, the process for their preparation and their use as antibiotics. The above mentioned substances are correlated with antibiotic A/16686 and are produced by the same microorganism *Actinoplanes* sp. ATCC 33076 which produces the above mentioned antibiotic.

It was then found that three closely related components could be isolated from antibiotic A₁₆₆₈₆ which were named factor A₁, A₂ and A₃. Factor A₂ (ramoplanin) is the component obtained in preponderant amount and is the most relevant for the biological activity, while factors A₁ and A₃ are obtained in a minor

amount. These substances as well as their preparation and uses are described in U.S. Patent 4,427,656.

A method for selectively enhancing the production of factors A2 and/or A3 of antibiotic A/16686 by adding appropriate precursors to an A/16686 producing culture is described in European Patent Application Publication No. 0259780.

5 The three compounds of this invention, which for the sake of brevity, are identified also as A/16686 factor A'1 ($R = -CO-CH=CH-CH=CH-CH_2-CH_2-CH_3$), factor A'2 ($R = -CO-CH=CH-CH=CH-CH_2-CH(CH_3)_2$) and factor A'3 ($R = -CO-CH=CH-CH=CH-CH_2-CH_2-CH(CH_3)_2$), respectively, may be produced by fermentation of *Actinoplanes* sp. ATCC 33076, a strain which has been deposited with the permanent culture collection ATCC as described in U.S. Patent 4,303,646 and is now freely available and accepted
10 under the Budapest Treaty as of January 31, 1981, or a producing mutant thereof (i.e., natural or artificial mutant capable of producing the same substances) under submerged aerobic conditions, in an aqueous nutrient medium containing assimilable sources of carbon and nitrogen and inorganic salts. To produce workable amounts (in this case, the term "workable amounts" is intended to mean that the amount of the invention compounds contained in the crude isolate from the fermentation broth is sufficient to permit their
15 isolation with the usual separation and purification techniques in a quantity suitable for experimental purposes and practical utilization) of the three above mentioned compounds in the crude fermentation product, the fermentation media must contain appropriate sources of the essential elements mentioned above.

Preferred carbon sources are sugars such as dextrose, fructose, maltose, sucrose and the like, polyols
20 such as glycerol and the like, starch and modified starches such as dextrin, with sucrose, glycerol and dextrin being the most preferred. However, it appears that when the concentration of dextrose in the nutrient culture medium is moderate or low, this has a positive effect on the formation of the compounds of formula I as it can be inferred from their larger proportion in the crude isolate from the fermentation broth.

Preferred nitrogen sources are soybean meal, peptone, tryptone, malt extract, yeast extract, aminoacids
25 and the like with soybean meal and malt extract being the most preferred.

Among the inorganic salts which are usually incorporated in the culture media are the customary soluble salts capable of yielding sodium, potassium, iron, zinc, cobalt, manganese, magnesium, calcium, ammonium, chloride, iodide, carbonate, sulfate, phosphate, nitrate ions and the like.

Addition of appropriate precursors during the fermentation according to the procedure described in
30 European Patent Application Publication No. 0259780 may be useful to selectively increase the ratio of one of the new antibiotics of formula I over the others. For instance, addition of from 0.2 g/l to 5 g/l of leucine (or its salts) increases the ratio of factor A'2 relative to factors A'1 and A'3 while addition of from 0.2 g/l to 5 g/l of valine (or its salts) selectively increases the relative ratio of factor A'3. Isovaleric acid has the same effect as leucine while isobutyric acid has the same effect as valine.

Ordinarily, the antibiotic-producing strain is pre-cultured in a shake flask, then the culture is used to inoculate jar fermentors for production of substantial quantities of the antibiotic substances. The medium used for the pre-culture can be the same as that employed for larger fermentations, but other media can also be employed. The producing-strain can be grown at temperatures between 20 and 40°C, preferably
35 between 24 and 35°C, most preferably between 28 and 32°C for a period of time (generally varying from 30 to 180 hours) during which an increase of antibiotic activity is observed.

During fermentation, the antibiotic production can be monitored by testing broth or mycelial extract samples for antibiotic activity, for instance, by bioassays or TLC or HPLC procedures.

Sensitive organisms to the antibiotics of this invention such as *Bacillus subtilis* or *Bacillus pumilus* can be used as test organisms. The bioassay is conveniently performed by the agar diffusion method on agar
45 plates.

Maximum production of antibiotic activity generally occurs between the third and the fifth day after inoculation.

The antibiotics produced during fermentation of the strain *Actinoplanes* sp. ATCC 33076 are found mainly in the mycelial mass. Therefore, the antibiotics of this invention are suitably recovered by separating
50 the mycelium from the fermentation broth, extracting the mycelial mass with an appropriate solvent system, isolating the crude fermentation product from said extract, separating and purifying the above antibiotic substances from the isolated crude fermentation product. A preferred method of recovering the A/16686 factor A'1, A'2 and A'3 antibiotics comprises extraction of the wet mycelium after separation from the fermentation broth by filtration at a pH between 4.5 and 5.5.

55 Extraction of the mycelial mass is best accomplished with water miscible organic solvents such lower alkanols, acetone and their mixtures at a pH between 1.5 and 2.5.

A crude fermentation product (the "crude"), which is a mixture of A/16686 antibiotics, i.e. factor A1, A2 and A3 (U.S. Patent 4,427,656) admixed with compounds of this invention, i.e., factor A'1, A'2 and A'3 together

with impurities, side products, salts, filter aids and materials deriving from the fermentation broth components, is recovered from the extracting solvent(s) by routine procedures.

The isolation of the antibiotic substances of this invention from the crude fermentation product, their separation and purification is conducted according to known per se techniques which include extraction with solvents, precipitation by adding non-solvents or by changing the pH of the solution, partition chromatography, reverse-phase partition chromatography, ion-exchange chromatography, affinity chromatography, HPLC techniques and the like.

According to a typical method of recovering the "crude", the mycelial extract in a mixture of water and water miscible organic solvents at acidic pH, which contains the above antibiotics in the form of acid addition salt, is usually extracted with an organic solvent poorly miscible with water and having a high dissolving power for lipophilic compounds (e.g., ethyl acetate, ethyl ether, n-hexane) and then the aqueous layer is concentrated under vacuum. The concentrated solution is then brought to pH 7 by addition of diluted alkali or ammonia and then filtered or centrifuged to recover the solid. Filter aids can be also added to the mixture to favor the recovery of the solid product.

The wet "crude" cake obtained can be further elaborated for isolation and purification of the compounds of formula I above. If a filter aid has been added before filtration it may be necessary to submit the "crude" cake to a further extraction with a mixture of water and a water miscible organic solvent at acidic pH like to the one applied for the extraction of the mycelial cake.

Re-precipitation of the "crude" from this extract is carried out by decreasing the solubility of the acid addition salts of the invention products in the extract by adding thereto a water miscible organic solvent wherein said acid addition salts are poorly soluble. Acetone and isopropanol are suitable organic solvents for said purpose.

The wet crude isolate is then further purified for eliminating therefrom most of the non-A/16686 antibiotic products. Thus, sludging the crude wet cake with a diluted (5 to 10% w/v) solution of a strong mineral acid followed by centrifugation is useful for eliminating some of the impurities. The wet cake thus obtained may be further purified by re-dissolving in a mixture of water and water soluble organic solvent of the same type as those employed for the extraction of the mycelial cake at a pH between 1.5 and 2.5. During this operation decolorizing agents can be added to the aqueous solution. Precipitation of the purified fermentation product containing the compound of formula I from the acidic solution may be carried out according to the procedure described above. Such purified preparation of the fermentation product is essentially consisting of a mixture of the compounds of this invention together with one or more other similar products, i.e., the factors A1, A2 and A3 of antibiotic A/16686. Isolation of the compounds of this invention from the above described mixture is carried out by the usual separation techniques as mentioned above. Preparative HPLC is particularly useful for both separation and purification purposes when the fermentation products contain considerable amount of antibiotic A/16686 factors A1, A2 and A3. The preparative HPLC operations are usually conducted under conditions which are common to the separation and purification of the A/16686 antibiotic. Examples of said separation and purification operations can be found, for instance, in U.S. Patent 4,427,656 where a C-18 alkyl silanized silicagel column and an eluent mixture of aqueous ammonium formate and acetonitrile are employed.

During the preparative HPLC the eluted liquids from each injection are checked by analytical HPLC and those fractions enriched in A/16686 factor A'1, A'2 and A'3, respectively, are separated.

The fractions enriched in each of the above compounds are combined and concentrated to dryness under vacuum. The respective solid residues are re-submitted to preparative HPLC under the same conditions as before. The solid products resulting from concentration of the eluted solutions are dissolved in diluted mineral acids and freeze-dried to yield the respective pure products under the form of mineral acid addition salts.

An alternative method for producing the compounds of this invention consists in contacting a substrate containing antibiotic A/16686 factor A1, A2 and A3 or a mixture thereof with the mycelium of Actinoplanes sp. ATCC 33076 or a natural or artificial mutant thereof capable of producing A/16686 antibiotics thus promoting the biotransformation into factors A'1, A'2 and A'3. According to this procedure, the single factors A1, A2 and A3 or a mixture thereof, such as, for instance, the complex resulting from a fermentation operation, are allowed to contact the mycelium for a period of time ranging from 50 to 200 hours at a temperature between 28 and 35 °C, preferably between 29 and 33 °C. This operation may be sequential to the fermentation of Actinoplanes sp. ATCC 33076 (or a producing mutant thereof) for producing the A/16686 antibiotics and therefore may consist in a prolonged contact of the fermentation product with the mycelium. When the fermentation operations have been carried out according to the conditions described above, the fermentation product already contains some amounts of factor A'1, A'2 and A'3 and, therefore, a prolonged contact with the mycelium actually results in an enrichment of the ratios of said compounds in the

fermentation batch.

According to a practical embodiment of this method, after the fermentation cycle is complete, i.e., when analytical tests show that the antibiotic activity in the fermentation broth is no longer increasing, inlet of oxygen (or air) into the fermentors is stopped and the fermentation broth is kept with stirring at a temperature between 29 and 33 °C for an additional period of time varying from 50 to 200 hours before harvesting under analytical HPLC control.

A preferred mode to carry out the biotransformation of the factor A1, A2 and A3 into the corresponding factor A'1, A'2 and A'3 consists in contacting the first three substances mentioned above (or a mixture thereof, including the A/16686 complex as resulting from the recovery operations from the fermentation batch, as described, for instance, in U.S. Patent 4,303,646) with the isolated mycelium of Actinoplanes sp. ATCC 33076 or a producing natural or artificial mutant thereof. Mycelium grown in a suitable culture medium is isolated from the fermentation broth (e.g. by centrifugation) and then added to a solution of factor A1, A2 or A3 or a mixture thereof in water or in a mixture of water and one or more organic solvents mixable with water (e.g. lower alkanols or acetone) with stirring. The temperature of the reaction mixture is maintained within the range indicated above while the pH may be kept steady around 7 by buffering the solution with a suitable buffer (e.g. a phosphate buffer). The biological transformation is usually followed by analytical HPLC. Experimental tests show that an A/16686 antibiotic crude mixture originally containing respectively about 14 percent of factor A1, about 64 percent of factor A2 and about 12 percent of factor A3 after about 140 hours of contact with the isolated mycelium of Actinoplanes sp. ATCC 33076 under the above conditions is transformed into a mixture containing respectively about 10% factor A'1, about 38% factor A'2 and about 5% factor A'3 with a concomitant decrease of the content of factor A1, A2 and A3 to 10%, 28% and 9% (HPLC areas), respectively. Analogously, a substrate consisting of an almost pure sample of factor A2 (HPLC titre: 89%) after 180 hours is converted into a mixture containing about 60% factor A'2 with the content of factor A2 decreasing to about 33% (HPLC areas).

The products resulting from the above described biotransformation are recovered from the reaction mixture (or from the fermentation broth, in case the biological transformation is directly operated by prolonging the contact with the mycelium in the fermentation batch) by acidifying the mycelial suspension to a pH between 1.5 and 2.5 and then adding thereto a water soluble organic solvent such as a lower alkanol or acetone, if this is not already present in a sufficient amount, to help extraction of the reaction products from the mycelial mass. After separation of the mycelium by filtration or centrifugation, the solution is elaborated in the same way as described above for isolating and purifying the compounds of this invention.

When the biological transformation is directly operated on the fermentation broth as mentioned above, the recovery of the reaction products can be carried out exactly as described before, for the recovery of the fermentation products.

Antibiotic A/16686 factor A'1, A'2 and A'3 are submitted to sugar content determination (acid hydrolysis), acid/base titration, amino acid analysis (for quantity and sequence), IR, UV, NMR spectrometry and Fast Atom Bombardment Mass Spectrometry (FAB-MS). The data resulting from these analytical tests confirm the assigned structures.

As shown in formula I the antibiotic substances of this invention possess basic functions which can form acid addition salts according to conventional procedures.

Representative and suitable acid addition salts of the compounds of formula I include those salts formed by standard reactions with both organic and inorganic acids such as, for example, hydrochloric, hydrobromic, sulfuric, phosphoric, acetic, trifluoroacetic, trichloroacetic, succinic, citric, ascorbic, lactic, maleic, fumaric, palmitic, cholic, pantoic, mucic, glutamic, camphoric, glutaric, glycolic, phthalic, tartaric, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic, cinnamic and the like.

The transformation of the free amino or non-salt compounds of the invention into the corresponding addition salts, and the reverse, i.e., the transformation of an addition salt of a compound of the invention into the non-salt form, are within the ordinary technical skill and are encompassed by the present invention.

For instance, a compound of the invention can be transformed into the corresponding acid addition-salt by dissolving the non-salt form in an aqueous solvent and adding a slight molar excess of the selected acid. The resulting solution or suspension is then lyophilized to recover the desired salt.

In case the final salt is insoluble in a solvent where the non-salt form is soluble it is recovered by filtration from the organic solution of the non-salt form after addition of the stoichiometric amount or a slight molar excess of the selected acid.

The non-salt form can be prepared from a corresponding acid salt dissolved in an aqueous solvent which is then neutralized to set free the non-salt form.

When following the neutralization desalting is necessary, a common desalting procedure may be

employed.

For example, column chromatography on silanized silica gel, non-functionalized polystyrene, acrylic and controlled pore polydextrane resins (such as Sephadex LH 20) or activated carbon may be conveniently used. After eluting the undesired salts with an aqueous solution, the desired product is eluted by means of a linear gradient or a step-gradient of a mixture of water and a polar or apolar organic solvent, such as acetonitrile:water from 50:50 to about 100% acetonitrile.

As it is known in the art, the salt formation either with pharmaceutically acceptable acids or non-pharmaceutically acceptable acids may be used as a convenient purification technique. After formation and isolation, the salt form of an antibiotic of formula I above can be transformed into the corresponding non-salt or into a pharmaceutically acceptable salt.

Antibiotic A/16686 factor A'1, A'2 and A'3 are particularly active against gram-positive microorganisms. The microbiological activity spectrum of antibiotic A/16686 factor A'2 is reported in the following Table:

TABLE I

| In vitro activity of A/16686 factor A'2 | |
|--|-----------------|
| Strain | MIC (mcg/ml) |
| <i>Staphylococcus aureus</i> Tour | 1 |
| <i>Staphylococcus aureus</i> Tour ^{a)} | 2 |
| <i>Staphylococcus aureus</i> Tour ^{b)} | 1 |
| <i>Staphylococcus epidermidis</i> ATCC 12228 | 0.25 |
| <i>Staphylococcus haemolyticus</i> L 602 ^{c)} | 1 |
| <i>Streptococcus pyogenes</i> C203 | 0.008 |
| <i>Streptococcus pneumoniae</i> UC41 | 0.008 |
| <i>Streptococcus faecalis</i> ATCC 7080 | 0.5 |
| <i>Streptococcus mitis</i> L 796 ^{c)} | 0.032 |
| <i>Clostridium perfringens</i> ISS 30543 | 2 |
| <i>Clostridium difficile</i> ATCC 9689 | 1 |
| <i>Propionibacterium acnes</i> ATCC 6919 | 0.5 |
| <i>Propionibacterium acnes</i> ATCC 6922 | 0.125 |
| <i>Propionibacterium acnes</i> L 1557 ^{c)} | 0.125 |
| <i>Propionibacterium acnes</i> L 1559 ^{c)} | 0.125 |
| <i>Propionibacterium acnes</i> L 1563 ^{c)} | 0.125 |
| <i>Propionibacterium acnes</i> L 1565 ^{c)} | 0.125 |

^{a)} Inoculum 10⁶ cfu/ml

^{b)} 30% bovine serum added

^{c)} Clinical isolates

Minimal Inhibitory Concentration (MIC) is determined by either the broth or the agar serial two-fold dilution method. Culture media and growth conditions: Iso-Sensitest broth (Oxoid), for staphylococci and *Streptococcus faecalis*; Todd-Hewitt broth (Difco), for other streptococcal species; Wilkins-Chalgren agar for anaerobic bacteria (T. D. Wilkins, S. Chalgren: Antimicrob. Agents Chemother. 10, 926 (1976)); the final inoculum is of about 10⁴ colony-forming units/ml or spot. MIC is read as the lowest concentration which shows no visible growth after 18-24 hours incubation at 37° C; for anaerobes the incubation is at 37° C for 48 hours in anaerobic atmosphere (N₂:CO₂:H₂, 80:10:10). The acute toxicity of factor A'2 is determined in CD1 mice (Charles River) of both sexes weighing 18-22 g by a single i.v. injection of the product, solubilized in sterile saline. Infusion rate is 0.15 ml/second. Dose levels are 75, 100, 125 and 150 mg/kg. The LD₅₀ value calculated is 103 mg/kg.

The other two products show biological activities comparable with that of antibiotic A/16686 factor A'2.

The antibiotic compounds of this invention are useful for preparing medicaments against infections primarily due to gram-positive widely diffused bacteria. In particular, the compounds of this invention are useful for topical treatment of wound infections and acne.

For use as medicaments the compounds of this invention can be administered by different routes either in the form of free compounds or in the form of their addition salts with pharmaceutically acceptable acids, this latter form being preferred. The topical route is usually the most suitable way to administer the

compounds of this invention.

For the medical uses the compounds of this invention are incorporated into pharmaceutical dosage forms suitable for oral, topical or parenteral administration such as tablets, capsules, lozenges, gelules, granules, powders, ointments, gels, liquid solutions, creams, solutions for injections, suspensions and the like. For instances, the formulations of said dosage forms can be carried out according to the general teaching of Remington's Pharmaceutical Sciences 17th Edition, 1985 Merck Publishing Company, Easton Pennsylvania.

The dosage unit may contain from 0.01 to 99 percent preferably from 0.5 to 80 percent of active ingredient. The daily dosage may depend on several factors such as body weight, the infecting microorganism, the severity of the infection, the age of the patient, the period and the way of administration. In general, the compounds of this invention are effective at a daily dosage ranging from about 2 mg to about 100 mg per kilogram of body weight, optionally divided into one or more administrations per day. Obviously, these dosages are only indicative and the most appropriate dosage can be adjusted in the specific cases and applications by relying on biological testings useful for determining the amount of active compound required to produced the desired effect.

The following Examples have the purpose to illustrate the invention but should not be construed as a limitation of its scope.

EXAMPLES

Example 1 - Fermentation of Actinoplanes sp. ATCC 33076 and recovery of the crude fermentation product

1.1 Fermentation of Actinoplanes sp. ATCC 33076

A lyophilized vial of master stock is used to inoculate two 18 cm oat meal agar slants. After incubation for one week at 29 °C, the mycelium of one slant is inoculated into a baffled flask of 500 ml containing 100 ml vegetative medium ⁽¹⁾, which is incubated at 29 °C with rotary agitation (200 r.p.m.).

After 48 hours the grown culture of one flask with pH = 7.0 is transferred to a 7-liter vessel with 4 liters of vegetative medium ⁽¹⁾ (900 r.p.m.; 28 °C; 0.5 v/v.m of air). After 48 hours, 25 ml of the tank culture, having pH = 7.1, are dispensed into 50 ml ampoules. The ampoules are then frozen in a slurry of acetone and dry ice and stored at -74 °C.

The frozen mycelium of 10 ampoules is inoculated in 10 baffled flasks (2000/400 ml) of vegetative medium ⁽¹⁾. After 51 hours of incubation at 29 °C with rotary agitation (150 r.p.m) the flasks have an average pH of 6.8. Subsequently, all 10 flasks are inoculated into a 10,500 l fermentor filled with 4000 l of vegetative medium ⁽¹⁾ sterilized in batch at 120 °C for 20 minutes.

After 68 hours at 29 °C the preculture, which has a pH of 6.8, is used to inoculate two 27,500 l fermentors filled with 20,000 l each of fermentation medium ⁽²⁾ sterilized in batch at 120 °C for 20 minutes. The fermentation temperature is kept constant at 29 °C and the fermentation course is followed by analytical HPLC and stopped after 65 hours.

| | |
|---|--------|
| (1): Composition of vegetative medium | |
| Soybean meal | 13 g/l |
| Dextrose | 12 g/l |
| Dextrin | 13 g/l |
| Calcium carbonate | 4 g/l |
| (2): Composition of fermentation medium | |
| Soybean meal | 30 g/l |
| Glycerol | 20 g/l |
| Dextrose | 4 g/l |
| Dextrin | 4 g/l |
| Crude malt extract | 20 g/l |
| Sucrose | 20 g/l |
| Calcium carbonate | 6 g/l |

1.2 Recovery of the crude fermentation product

The harvest broth from the two fermentors is cooled at 10 °C and brought to pH 5 with 20% (w/v) HCl and filtered on a rotary filter.

The wet mycelium is separated and extracted with 12,500 l of acetone in three times by stirring the suspension at pH 2 (with 30% HCl) and centrifuging. The hydroacetonetic extract is divided in four portions and extracted with a total volume of 12,600 l of ethyl acetate. The aqueous phase is concentrated under vacuum at 18 °C (internal temperature) to 7,000 l.

To the concentrated solution cooled at 4 °C, 150 l of 15% NH₄OH is slowly added until pH 7, in the presence of 210 l of CH₂Cl₂. After addition of 55 kg of filter aid the suspension is filtered on a rotary filter. The wet cake is re-extracted three times by using every time a mixture of 230 l of acetone and 44 l of water at pH 2 (20% HCl) with slurry at room temperature.

After washing the solid with a further portion of 110 l of a mixture acetone:water 6:4 at pH 2, the three solutions and washings are combined and 2,600 l of acetone is added thereto with stirring at room temperature. After standing overnight, the solid precipitate is centrifuged, washed with 240 l of acetone and dried at room temperature under vacuum yielding 54 kg of crude fermentation product having a 28% total A 16686 antibiotic HPLC titre.

Example 2 - Isolation and purification of the antibiotic A/16686 factors A'1, A'2 and A'3

2.1 Purified mixture of A/16686 antibiotics

A portion of 13 kg of the crude fermentation product obtained according to Example 1 is sludged with 80 l of 5% (w/v) HCl at about 15 °C by vigorous stirring for 3 hours. The suspension is centrifuged and the solid is washed with 24 l of 5% HCl. The wet cake is extracted with a mixture of 80 l of acetone and 33 l of water in the presence of 10.5 kg of decolorizing diatomaceous earth (Tonsil Optimum NFF, Süd-Chemie A.G., München) and 2.1 kg of charcoal (Darco G60) at 15 °C. The suspension is centrifuged and the solid is washed with 30 l of a mixture acetone:water 6:4. The extracts are combined (140 l in total) and 350 l of acetone is added slowly thereto with stirring. After standing overnight the mixture is filtered on a series of buckner filters. The resulting cake is washed with 10 l of a mixture acetone:water 9:1 and then with 4 l of acetone. The solid product remaining on the filter is dried under vacuum for 72 hours at room temperature to yield 1.86 kg of a purified preparation (89% HPLC titre) of A/16686 antibiotics which are used for further elaborations to isolate the compounds of this invention.

2.2 Isolation and purification of antibiotic A/16686 factor A'1, A'2 and A'3

27 Grams of the preparation obtained according to paragraph 2.1 is submitted to preparative HPLC by using the following apparatus and conditions for each portion of 300 mg dissolved in 5 ml of water containing 10% (v.v) of CH₃CN.

- 5 Instrument: The apparatus is set up by assembling a Waters mod. 590 pump, a Waters Lambda-Max mod. 481LC detector set at 285 nm, and a Rheodyne injector equipped with a 5 ml loop.
 Column: LiChrosorb RP-18, 10 micron, 250 mm x 50 mm (Merck)
 Mobile phase: 0.05 M HCOONH₄:CH₃CN (64:36)
 Flow rate: 30 ml/min

10

The operations are monitored by analytical HPLC (see Example 5, paragraph 5.1). The group of fractions enriched respectively in factor A'1, factor A'2 and factor A'3 are separated and each group of fractions is combined to form three solutions which are worked in the same manner as follows. The solution is concentrated to dryness under vacuum and the residue is purified again by preparative HPLC. The fractions containing the pure component are pooled and concentrated under vacuum. The solid residue is taken up twice with ethanol and collected by filtration. The dry solid is dissolved in 0.1N HCl and freeze-dried to yield the hydrochloride of the pure component which is submitted to analysis and physico-chemical characterization.

15 From the above mentioned sample the following amounts of pure hydrochlorides of factor A'1, A'2 and A'3 are respectively obtained: 150 mg, 600 mg and 30 mg.

20

Example 3 - Biological transformation of antibiotic A/16686 factor A2 into factor A'2

25 A sample (600 mg) of antibiotic A/16686 factor A2 (HPLC titre 89%) prepared by following the procedure described in U.S. Patent 4,427,656 is dissolved in 200 ml of water (in a 1000 ml flask). To this solution is added the mycelium of *Actinoplanes* sp. ATCC 33076 obtained by centrifugation of 400 ml of a culture grown in vegetative medium in a 7 liter vessel (as described under Example 1, paragraph 1.1 for preparing the inoculation mycelium).

30 The mixture is stirred at 200 r.p.m. at 32° C and the biological transformation is followed by analytical HPLC (see under Example 5, paragraph 5.1).

The HPLC analysis of the mycelial suspension is carried out every 2 hours. The analytical pattern show that the mycelial suspension which originally contains factor A2 in a concentration of 2053 micrograms/ml after 180 hours contains factor A2 and factor A'2 in concentrations of 282 micrograms/ml (33%) and 513 micrograms/ml (60%), respectively.

35 The suspension is worked up in the same manner as the fermentation broth in Example 1, first part of paragraph 1.1, to obtain a hydroacetic extract of the mixture of antibiotic A/16686 factor A2 and factor A'2 which is then precipitated from said extract by adding acetone in a proportion of about 3 volumes for each volume of hydroacetic extract.

40 The solid precipitate is then separated by filtration and worked up as in the last part of Example 2, paragraph 2.1, to yield a purified mixture of antibiotic A/16686 factor A2 and factor A'2 with an HPLC titre of about 33% and about 60%, respectively. Isolation and purification of factor A'2 from said mixture is carried out according to paragraph 2.2 of Example 2 yielding 55 mg of pure antibiotic A/16686 factor A'2 hydrochloride.

45

Example 4 - Biological transformation of a mixture of antibiotic A/16686 factors A1, A2 and A3 into a mixture containing factors A'1, A'2 and A'3

50 A sample of 600 mg of antibiotic A/16686 complex containing factor A1 13.9%, factor A2 64.3%, factor A3 12.3% (analytical HPLC) is submitted to the same procedure as described in Example 3. The reaction is stopped after 140 hours during which the reaction is monitored by HPLC. The analytical pattern of the mycelial suspension show that the concentration of factors A1, A2 and A3 which at the origin was 313 micrograms/ml, 1434 micrograms/ml and 227 micrograms/ml, respectively, after 140 hours has decreased to 96 micrograms/ml (10%), 273 micrograms/ml (29.4%) and 85 micrograms/ml (8.9%), respectively, while the concentration of factors A'1, A'2 and A'3 is 99 micrograms/ml (10.3 %), 364 micrograms/ml (37.9%) and 45 micrograms/ml (4.7%), respectively.

55 The suspension is worked out as described in Example 3 above and to yield a purified mixture of

A.16686 antibiotics with the following HPLC titre: factor A1 (10%), factor A2 (28%), factor A3 (9%), factor A'1 (10%), factor A'2 (38), factor A'3 (5%). From this preparation factor A 1, A 2 and A 3 are isolated in the form of the respective hydrochlorides by following the same procedure described under Example 2, paragraph 2.2.

5

Example 5 - Analytical assays and physico-chemical characterization

5.1 Analytical HPLC

Apparatus: Hewlett-Packard liquid chromatograph, mod. 1084 B equipped with a UV detector set at 254 nm. Column: Erbasil C-18, 5 micron, 150 mm x 4.6 mm (Carlo Erba)

Mobile phase:

15 A) 0.05 M NaH_2PO_4 pH 4;

B) 0.05 M NaH_2PO_4 pH 4: CH_3CN 73:23

Flow rate: 0.5 ml/min

Gradient profile:

20

| | | | |
|-----|----|----|----|
| min | 0 | 10 | 40 |
| % B | 45 | 45 | 65 |

Injection volume: 20 microliters of a solution of the substance to be examined at about 0.5 mg/ml in water

25 Under these conditions the respective retention times (t_R) are as follows:

30

| | t_R (minutes) |
|------------|-----------------|
| factor A1 | 15.14 |
| factor A'1 | 17.81 |
| factor A2 | 20.30 |
| factor A'2 | 22.70 |
| factor A3 | 24.85 |
| factor A'3 | 27.24 |

35

When the assay is performed on the fermentation broth, the sample to be analyzed is prepared as follows:

40 two milliliters of the fermentation broth are drawn and 40 microliters of 6N HCl are added thereto to bring the pH value between 1.8 and 2.0. Two milliliters of acetone are added to the mixture which is stirred for ten minutes and then centrifuged for five minutes at 3000 r.p.m. The mycelium is separated and a portion of two milliliters of the solution is extracted with an equal volume of ethyl acetate. After separation of the two phases the aqueous layer is separated (about 1.2 ml) and used for HPLC determination.

45

5.2 Sugar analysis

50 Sugar determination is made after acid hydrolysis (2N H_2SO_4 , 100°C, 2 hours) as described by B. Cavalleri et al. in The Journal of Antibiotics, Vol. 37, No. 4, pp 309-317, 1984. The presence of one D-mannose unit for each molecule of the three factors A'1, A'2 and A'3 is observed.

5.3 Amino acid analysis and $^1\text{H-NMR}$ spectra

55

The acid hydrolysis is performed on all three compounds with 6N HCl at 105°C for 20 hours in a sealed tube. The mixture of amino acids is separated by column chromatography on a strongly acidic sulfonic divinylbenzene resin (Dowex 50 W) by eluting with aqueous HCl of increasing concentrations from

0.5N to 2N.

The amino acids are identified by comparison with authentic samples on the basis of ¹H-NMR and GC-MS. The amino acid ratio and their sequence in the intact molecules are determined by NMR experiments.

All three compounds show the same amino acids compositions and sequence.

- 5 The following Table II shows the type and number of amino acid residues in each of the three factors A'1, A'2 and A'3.

Table II

| Amino acid | Number of units |
|---------------------------------|-----------------|
| Threo-beta-hydroxyaspartic acid | 1 |
| Aspartic acid | 1 |
| 15 Allo-threonine | 3 |
| Glycine | 1 |
| Alanine | 1 |
| 4-Hydroxyphenylglycine | 5 |
| Leucine | 1 |
| 20 Phenylalanine | 1 |
| 3-Chloro-4-hydroxyphenylglycine | 1 |
| Ornithine | 2 |

25 Two equivalents of ammonia per mole of each factor is titrated on the respective acid hydrolysis mixture by means of an amino acid automatic analyzer providing evidence of two primary amide groups.

Furthermore, the total number of amidic nitrogen atoms (19) resulting from ¹⁵N NMR experiments exceed by two the number of nitrogen atoms involved in the peptide bonds according to the number of amino acids in the molecule (Table II). Titration of the factors does not show any presence of free carboxylic groups. These considerations support that the two primary amide groups are on the aspartic and threo-beta-hydroxyaspartic acid units, respectively.

30 The NMR spectra are recorded in the temperature range from 25 to 60 °C on a Bruker AM 250 spectrometer equipped with an Aspect 3000 computer.

Standard pulse sequence and standard software are used for the 2D-NMR spectra, with slight modifications to suppress the water peak during the measurement.

35 The following 2D techniques are used: COSY, Relayed COSY, (W.J. Chazin, D.P. Goldenberg, T.E. Creighton, K. Wüthrich: Eur.J. Biochem., 152, 429-437 (1985)), COSY with enhancement of long range couplings (A. Bax, R. Freeman: J. Magn. Reson., 44, 542-561 (1981)), NOESY (S. Macura, K. Wüthrich, R.R. Ernst: J. Magn. Reson. 47: 351-357 (1982)) and C¹³LOC (H.Kessler, C.Griesinger, J. Zarbock, H.R. Loosli: J. Magn. Reson. 57, 331-336 (1984)).

40 The following Table III shows the ¹H-NMR chemical shifts (delta, ppm) of the amino acids of the amino acidic moiety of antibiotic A/16686 factor A'2 in H₂O : DMSO, 4:1 at pH 4.6, temp. 40 °C, internal standard TMS (delta = 0.00 ppm)

TABLE III

| Amino acid | HN | HC _{alpha} | HC _{beta} | others |
|-----------------------------|------|---------------------|--------------------|----------------------------------|
| 1 Aspartic acid | 8.09 | 4.76 | 2.17, 1.62 | - |
| 2 Beta-hydroxyaspartic acid | 8.41 | 5.49 | 5.69 | - |
| 3 4-Hydroxyphenylglycine | 9.84 | 6.21 | - | Phenyl 7.64 (b,f), 7.02 (c,e) |
| 4 Ornithine | 9.20 | 4.21 | 2.04 | - |
| 5 Threonine | 7.56 | 4.36 | 4.03 | HC _{gamma} 1.04 |
| 6 4-Hydroxyphenylglycine | 8.91 | 5.44 | - | Phenyl 6.67 (b,f), 6.45 (c,e) |
| 7 4-Hydroxyphenylglycine | 8.92 | 6.78 | - | Phenyl 6.82 (b,f), 6.36 (c,e) |
| 8 Threonine | 8.24 | 3.71 | 3.92 | HC _{gamma} 0.82 |
| 9 Phenylalanine | 7.74 | 4.97 | 2.04 | Phenyl 7.22 (b,f), 6.92 (c,d,e,) |

TABLE III (continued)

| Aminoacid | HN | HC _{alpha} | HC _{beta} | others |
|----------------------------------|------|---------------------|--------------------|--|
| Ornithine | 7.59 | 4.20 | 2.18, 1.89 | HC _{gamma} 1.67, HC _{delta} 3.02 |
| 4-Hydroxyphenylglycine | 9.23 | 6.94 | - | Phenyl 7.34 (b,f), 6.95 (c,e) |
| Threonine | 9.06 | 4.72 | 3.94 | HC _{gamma} 0.95 |
| 4-Hydroxyphenylglycine | 8.78 | 6.09 | - | Phenyl 7.08 (b,f), 6.70 (c,e) |
| Glycine | 7.89 | 3.76, 2.99 | - | - |
| Leucine | 8.36 | 4.26 | 1.47 | HC _{gamma} 1.47, HC _{delta} 0.74 |
| Alanine | 9.32 | 4.32 | 1.41 | - |
| 3-Chloro-4-hydroxyphenyl-glycine | 7.87 | 4.91 | - | Phenyl 6.83 (f), 6.51 (b), 6.36 (e) |

Factors A'1 and A'3 show the same pattern.

Figure 1 reports the ¹H-NMR spectrum of factor A'2.

¹H-NMR spectra of factors A'1 and A'3 show the same pattern as that of factor A'2, apart from the
5 signals attributed to the fatty acid chains attached to the aspartic moiety.

5.4 Fatty acid moieties

10 The following Table IV summarizes the ¹H-NMR chemical shifts (delta, ppm) assignments of the fatty acid moiety of each of the three compounds in H₂O:DMSO, 4:1 at pH 4.6, temperature 40 °C, internal standard TMS (delta = 0.00 ppm).

TABLE IV

15

| Factor | HC-2 | HC-3 | HC-4 | HC-5 | HC-6 | Others |
|--------|------|------|------|------|------|--|
| A'1 | 5.52 | 6.25 | 7.26 | 6.12 | 2.00 | 1.67 (2H) HC-7; 0.85 (3H) HC-8 |
| A'2 | 5.52 | 6.25 | 7.26 | 6.12 | 2.00 | 1.67 (1H) HC-7; 0.81, 0.82 (6H) HC-8 and 7-Me |
| 20 A'3 | 5.52 | 6.25 | 7.26 | 6.12 | 2.00 | 1.67 (3H) HC-7 and HC-8; 0.75 (6H) HC-9 and 8-Me |

25 5.5 Sugar

The D-mannose unit gives the following ¹H-NMR signals (delta, ppm) for each of the three factors in H₂O:DMSO, 4:1 at pH 4.6, temperature 40 °C, internal standard TMS (delta = 0.00 ppm): 5.40 (one anomeric proton); 4.01, 3.91-3.71, 3.60-3.51 (other protons). The attachment point of the sugar to the
30 peptidic moiety is determined by NMR experiments (Nuclear Overhauser Effect).

5.6 Lactone ring

35 The presence of a lactone ring is supported by the absorbance at 1760 cm⁻¹ in the IR spectrum (see under Example 6, paragraph 6.1). The position of the lactone bond is established by

- identification of the amino acid contributing to the lactone bond with its carboxylic group
- identification of the hydroxy-amino acid contributing to the lactone bond with its hydroxyl group.

40 According to step a), finely ground CaCl₂ (2.5 g) is added to a cooled (0-5 °C) solution of NaBH₄ (1.5 g) in absolute EtOH (150 ml) with magnetic stirring. After 1.5 hour factor A'2 (1 g) dissolved in dry DMF (60 ml) is added dropwise. The cooled reaction mixture is stirred for 24 hours, then it is cautiously poured into water (600 ml). The pH is adjusted at 5 with aqueous HCl and the solution is desalted on a column filled with a macroporous cross-linked polystyrene resin XAD-2 (Amberlite Rohm and Haas) by eluting first with H₂O to remove salts and after with a mixture 0.01N HCl:CH₃CN 1:1 to recover the reduced peptide.
45 Fractions containing the peptide are combined and concentrated to dryness under vacuum.

The residue is hydrolyzed with 6N HCl at 105 °C for 20 hours. The acidic solution after extraction with ethyl acetate is concentrated under vacuum. The residue is chromatographed on a Dowex 50Wx4 column by eluting with 0.5N HCl. The fractions containing the wanted compound (checked by bidimensional HPTLC: cellulose, first run butanol:acetic acid:H₂O 4:1:5, upper layer, second run pyridine:H₂O 4:1; spots of amino acids are located by spraying with ninhydrin and heating at 120 °C for 5 minutes) are combined and concentrated. The oily residue is purified again by preparative layer chromatography (SiO₂, 5 mm thick, butanol:acetic acid:H₂O 4:1:5, upper layer) to obtain the 2-amino-2-(3-chloro-4-hydroxyphenyl)ethyl alcohol:
50 ¹H-NMR (250 MHz, DMSO-d₆) delta, ppm 3.45 (m, CH, partly covered by the water signal), 3.38 (m, CH₂), 6.92 (d, CH-5), 7.11 (dd, CH-6, ³J = 8.8 Hz), 7.32 (d, CH-2, ⁴J = 2.5 Hz). The structure is further confirmed by comparison with an authentic sample prepared from 3-chloro-4-hydroxyphenylglycine (30 mg) which is converted into its methyl ester (MeOH, HCl) then reduced with Ca(BH₄)₂ following the procedure reported above. Step b) is accomplished by first reacting factor A'2 with phenylisocyanate. This latter reacts
55 with all the free hydroxyl and amino groups of the peptide giving urethanes and ureas, respectively, that are

usually rather resistant to acid hydrolysis carried out, as described in Example 5, paragraph 3. The amount of hydroxyaspartic acid does not decrease, while the amounts of the other hydroxylated amino acids decrease, thus indicating that it is the amino acid involved in the lactone linkage with its hydroxyl group. Accordingly, factor A'2 (100 mg) is dissolved in DMF (2 ml) and phenylisocyanate (300 microliters) is added. The reaction mixture is left at room temperature for 48 hours, quenched by adding H₂O (20 ml) and filtered. The solid compound is hydrolyzed and analyzed for its amino acid composition as described in Example 5, paragraph 3.

The same experiments carried out with factors A'1 and A'3 give corresponding results.

Example 6 - IR, UV and FAB-MS spectra

6.1 The IR spectrum of factor A'2 recorded as nujol mull with a Perkin-Elmer mod. 580 spectrophotometer is shown in Fig. 2 of the accompanying drawings. The following absorption maxima are observed: 3500-3100 (ny NH and ny OH), 3020-2800 (nujol), 1760 (ny C=O lactone), 1630 (ny C=O, amide I), 1510 (delta NH, amide II), 1460 and 1375 (nujol), 1225 (ny C-O, lactone), 1065-980 (ny C-O, sugars), 840 and 815 cm⁻¹ (gamma CH aromatic)

The spectra of the other two factors do not show substantial differences.

6.2 The Ultraviolet Spectrum of factor A'2 registered in water with a Perkin Elmer mod. 320 spectrophotometer is given in Fig. 3 of the accompanying drawings. The spectrum exhibits the following absorption maxima: 232 nm (log epsilon 4.64), 270 nm (log epsilon 4.34). The spectra of the other two factors do not show substantial differences.

6.3 The Fast Atom Bombardment Mass Spectra (FAB-MS) are recorded with a MS9/50TC instrument using a thioglycerol/glycerol 1:1 mixture as a matrix. Bombardment gas Xe; kinetic energy 6 keV; accelerating voltage 4kV. The isotopic clusters of the cationized molecular ions MH⁺ indicate molecular weights of 2375 (factor A'1), 2389 (factor A'2) and 2403 (factor A'3), respectively, (lowest isotope compositions). These data, and the presence in the spectra of fragment ions corresponding to the loss of 163 u from the respective MH⁺ ions are in agreement with the structures assigned.

6.4 Elemental analysis

The elemental analysis, after the sample has been previously dried at about 140°C under inert atmosphere, give the following approximate percentage composition:

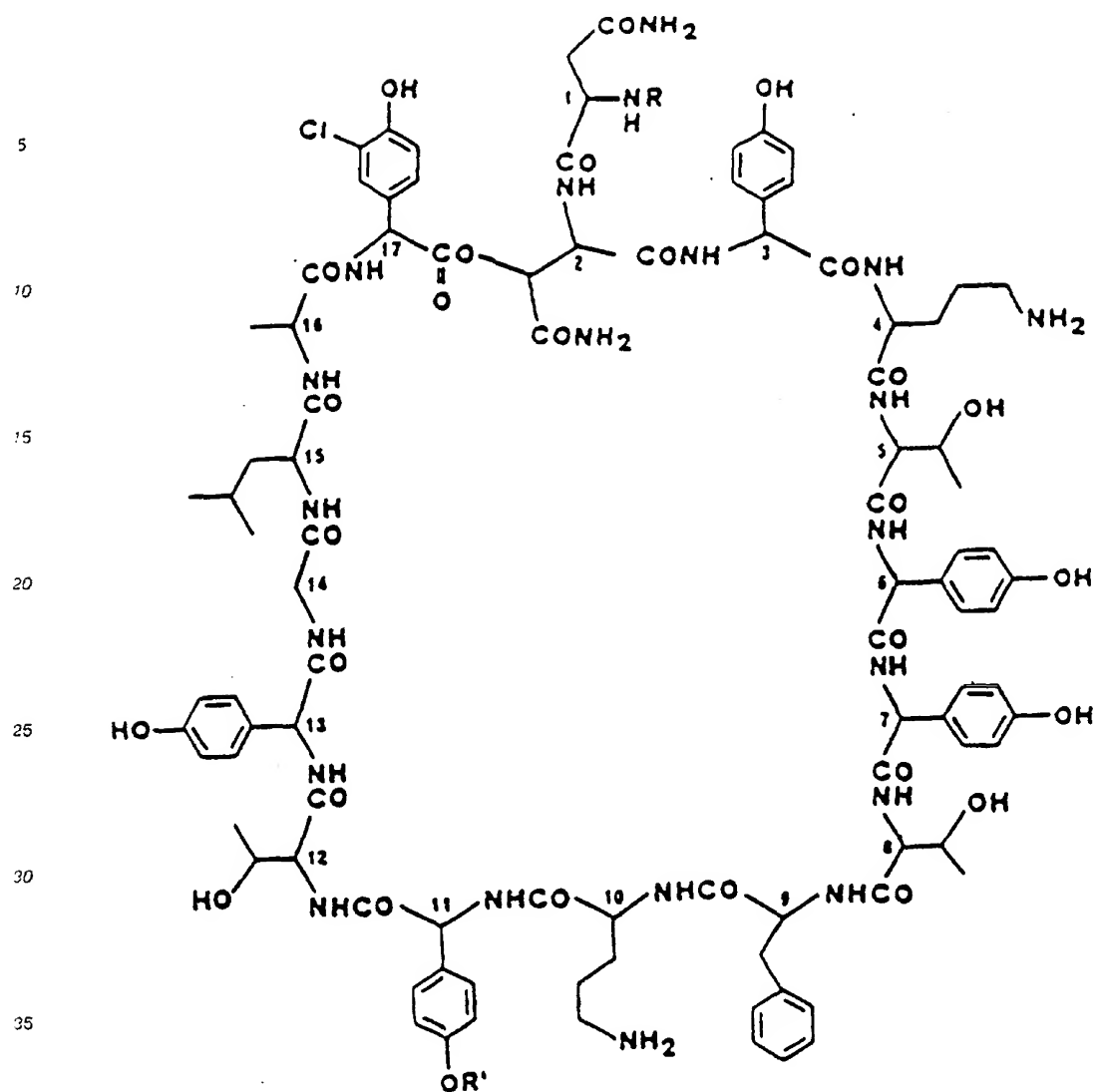
| | factor A'1 | factor A'2 | factor A'3 |
|---------|---------------|---------------|---------------|
| C % | 54.2 | 55.2 | 55.8 |
| H % | 5.8 | 5.4 | 6.2 |
| N % | 11.8 | 12.6 | 11.4 |
| Cl % | 3.9 | 4.2 | 4.0 |
| ashes % | 0.9 | 1.3 | 0.7 |

The above values are in agreement with those calculated for the respective di-hydrochloride salts of the three compounds.

Claims

CLAIMS FOR THE CONTRACTING STATES: AT BE CH DE FR GB IT LU NL SE

1) A compound of formula I



wherein:

R is: $-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_3$,

$-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}(\text{CH}_3)_2$ or

$-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{CH}_3)_2$

and R' is alpha-D-mannopyranosyl and the acid addition salts thereof.

2) A compound of claim 1 wherein R is

$-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_3$

3) A compound of claim 1 wherein R is

$-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}(\text{CH}_3)_2$

4) A compound of claim 1 wherein R is

$-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{CH}_3)_2$

5) An acid addition salt of claim 1 which is the dihydrochloride.

6) A process for producing the compounds of claim 1 characterized in that (a) the microorganism Actinoplanes sp. ATCC 33076 or a producing mutant thereof is fermented under submerged aerobic conditions in an aqueous medium containing assimilable sources of carbon and nitrogen and inorganic salts, (b) the mycelium is separated from the fermentation broth, (c) the mycelial mass is extracted with an appropriate solvent system, (d) the crude fermentation product is isolated from said extract and (e) the above antibiotic substances are separated and purified from the isolated crude fermentation product.

7) A process as in claim 6 wherein the carbon sources are selected from dextrose, fructose, maltose, sucrose, glycerol and dextrin, the nitrogen sources are selected from soybean meal and malt extract.

8) A process as in any of claims 6 and 7 wherein the fermentation is carried out at a temperature between 24 and 35 °C, preferably between 28 and 32 °C, for the period of time during which an increase of antibiotic activity is observed.

9) A process as in any of claims 6 through 8 wherein the mycelium is separated from the fermentation broth by filtration or centrifugation at a pH between 4.5 and 5.5, the mycelial mass is extracted with a water miscible organic solvent selected from lower alkanols, acetone and mixtures thereof at a pH between 1.5 and 2.5, and the crude fermentation product is isolated from the extract of the mycelial mass by adding thereto a water miscible organic solvent wherein the acid addition salts of the antibiotic substances are poorly soluble.

10) A process as in any of claims 6 through 9 wherein the separation and purification of the antibiotic substances from the isolated crude fermentation product is carried out by preparative HPLC methods.

11) A method for producing the compounds of claim 1 characterized in that a substrate containing antibiotic A/16686 factor A1, A2, and A3 or a mixture thereof is contacted with the mycelium of Actinoplanes sp. ATCC 33076 or a natural or artificial mutant thereof capable of producing A/16686 antibiotics.

12) A process as in claim 11 wherein the substrate is contacted with the mycelium at a pH around 7 at a temperature between 28 and 35 °C, preferably between 29 and 33 °C, for a period of time varying from 50 to 200 hours.

13) A process as in any of claims 11 and 12 wherein the mycelium is previously isolated from the fermentation broth and the contact with the substrate is carried out in a solution of water or a mixture of water with one or more water mixable organic solvents, preferably selected from lower alkanols and acetone.

14) A process as in any of claims 11 through 13 wherein the reaction products are separated from the reaction medium by acidifying the mycelial suspension at a pH between 1.5 and 2.1, adding thereto a water mixable organic solvent or a mixture of water mixable organic solvents whenever said solvent(s) is/are not already present in the reaction mixture in amount sufficient for extraction of the reaction product(s) from the mycelial mass, separating the mycelium by centrifugation or filtration and then treating the extract according to any of the claims 9 and 10.

15) A compound of claim 1 for use as antibacterial.

16) A pharmaceutical composition containing a compound of formula I of claim 1 or a pharmaceutically acceptable acid addition salt thereof.

17) Use of a compound of claim 1 for preparing a medicament for combatting infectious diseases due to gram-positive microorganisms.

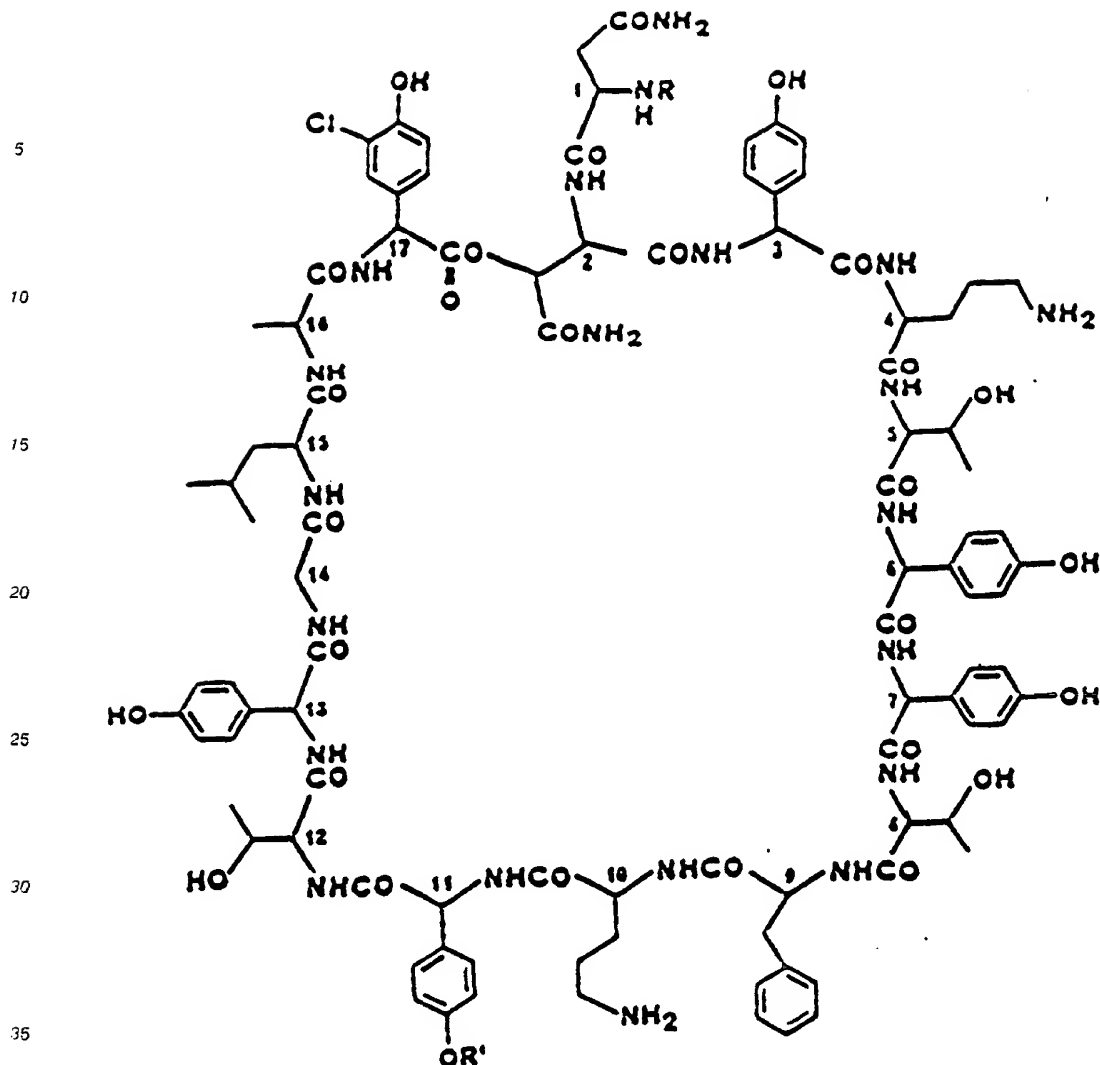
18) A compound of claim 1 for use in the treatment of wound infections or acne.

19) A pharmaceutical composition containing a compound of claim 1 as the active ingredient.

20) A pharmaceutical composition according to claim 19 particularly useful for the topical treatment of wound infections or acne.

CLAIMS FOR THE CONTRACTING STATES: GR, ES

1) A process for preparing a compound of formula I



wherein:

R is: $-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_3$,
 $-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}(\text{CH}_3)_2$ or
 $-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}(\text{CH}_3)_2$

and R' is alpha-D-mannopyranosyl and the acid addition salts thereof characterized in that (a) the microorganism *Actinoplanes* sp. ATCC 33076 or a producing mutant thereof is fermented under submerged aerobic conditions in an aqueous medium containing assimilable sources of carbon and nitrogen and inorganic salts, (b) the mycelium is separated from the fermentation broth, (c) the mycelial mass is extracted with an appropriate solvent system, (d) the crude fermentation product is isolated from said extract and (e) the above antibiotic substances are separated and purified from the isolated crude fermentation product.

2) A process as in claim 1 for preparing a compound of formula I wherein R is

$-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_2-\text{CH}_3$

3) A process as in claim 1 wherein R is

$-\text{CO}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}(\text{CH}_3)_2$

4) A process as in claim 1 wherein R is



5) A process as in any of claims 1 through 4 for preparing the compound of claim 1, 2, 3 or 4 in the form of an acid addition salt.

6) A process as in claim 5 wherein the acid addition salt is the dihydrochloride.

7) A process as in any of the claims 1 through 6 wherein the carbon sources are selected from dextrose, fructose, maltose, sucrose, glycerol and dextrin, the nitrogen sources are selected from soybean meal and malt extract.

8) A process as in any of claims 1 through 7 wherein the fermentation is carried out at a temperature between 24 and 35 °C, preferably between 28 and 32 °C, for the period of time during which an increase of antibiotic activity is observed.

9) A process as in any of claims 1 through 8 wherein the mycelium is separated from the fermentation broth by filtration or centrifugation at a pH between 4.5 and 5.5.

10) A process as in any of claims 1 through 9 wherein the mycelial mass is extracted with a water miscible organic solvent selected from lower alkanols, acetone and mixtures thereof at a pH between 1.5 and 2.5.

11) A process as in any of claims 1 through 10 wherein the crude fermentation product is isolated from the extract of the mycelial mass by adding thereto a water miscible organic solvent wherein the acid addition salts of the antibiotic substances are poorly soluble.

12) A process as in any of claims 1 through 11 wherein the separation and purification of the antibiotic substances from the isolated crude fermentation product is carried out by preparative HPLC methods.

13) A process as in claim 12 wherein the separation and purification procedures are carried out by employing a C-18 alkyl silanized silicagel column and an eluent mixture of aqueous ammonium formate and acetonitrile.

14) A process as in any of claims 1 through 13 wherein during the fermentation process a suitable precursor is added in order to selectively increase the ratio of one of the antibiotic substances of formula I over the others.

15) A process as in claim 14 wherein the precursor is selected from leucine, valine, isovaleric acid, isobutyric acid or a salt thereof.

16) A method for producing a compound as in claims 1 through 6 characterized in that a substrate containing antibiotic A/16686 factor A1, A2, and A3 or a mixture thereof is contacted with the mycelium of *Actinoplanes* sp. ATCC 33076 or a natural or artificial mutant thereof capable of producing A/16686 antibiotics.

17) A process as in claim 16 wherein the substrate is contacted with the mycelium at a pH around 7 at a temperature between 28 and 35 °C, preferably between 29 and 33 °C, for a period of time varying from 50 to 200 hours.

18) A process as in any of claims 16 and 17 wherein the mycelium is previously isolated from the fermentation broth and the contact with the substrate is carried out in a solution of water or a mixture of water with one or more water mixable organic solvents, preferably selected from lower alkanols and acetone.

19) A process as in any of claims 16 through 18 wherein the reaction products are separated from the reaction medium by acidifying the mycelial suspension at a pH between 1.5 and 2.1, adding thereto a water mixable organic solvent or a mixture of water mixable organic solvents whenever said solvent(s) is/are not already present in the reaction mixture in amount sufficient for extraction of the reaction product(s) from the mycelial mass, separating the mycelium by centrifugation or filtration and then treating the extract according to any of the claims 11 through 13.

20) A process as in claim 16 wherein the contact between the substrate and the mycelium is carried out directly in the fermentation broth, by prolonging the contact of the fermentation product with the mycelium until substantial transformation of A factors to A factors occurs and the recovery of the reaction products is carried out according to claims 9 through 13.

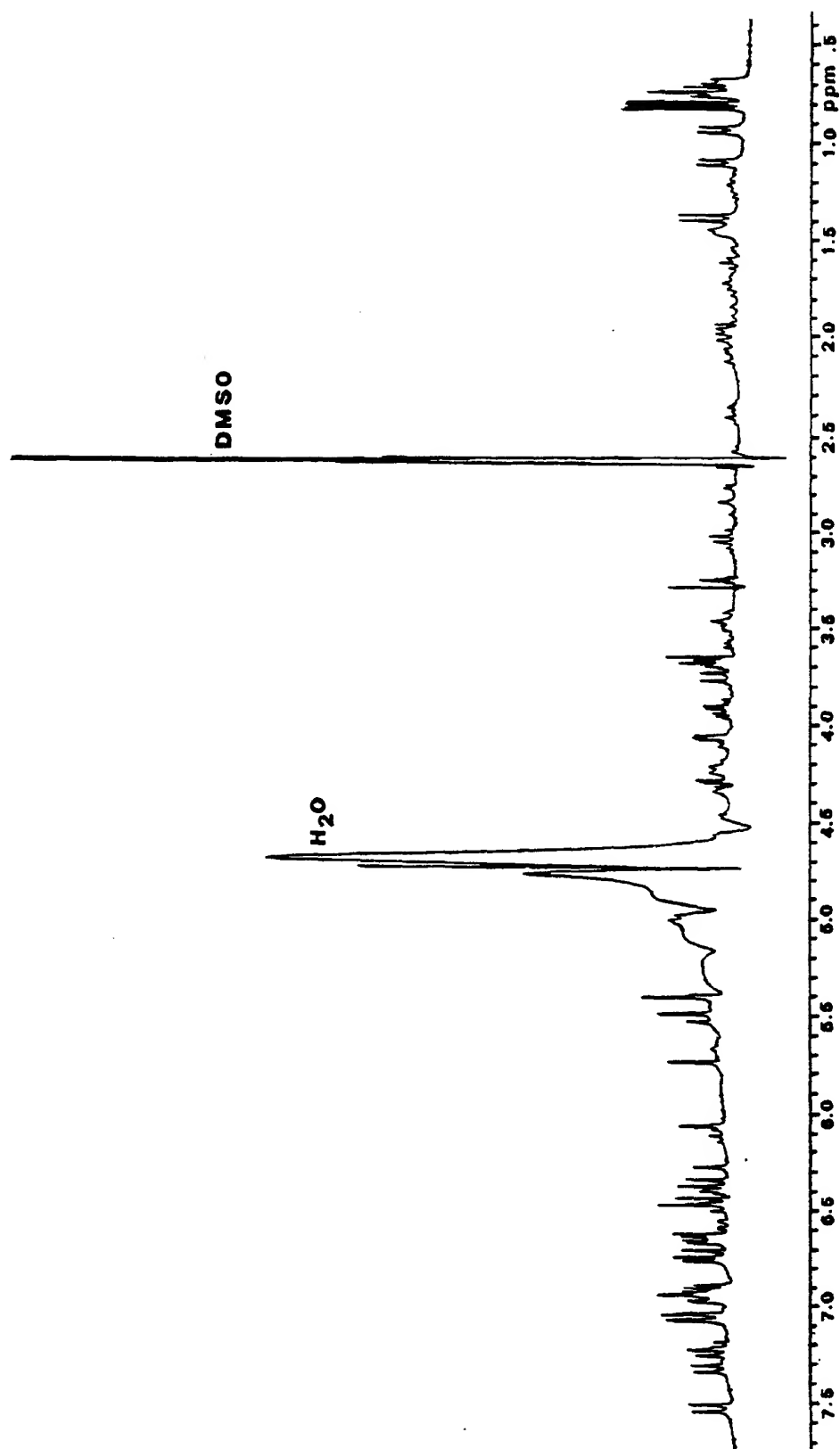
^1H -NMR SPECTRUM OF FACTOR A'2

FIG. 1

IR SPECTRUM OF FACTOR A'2

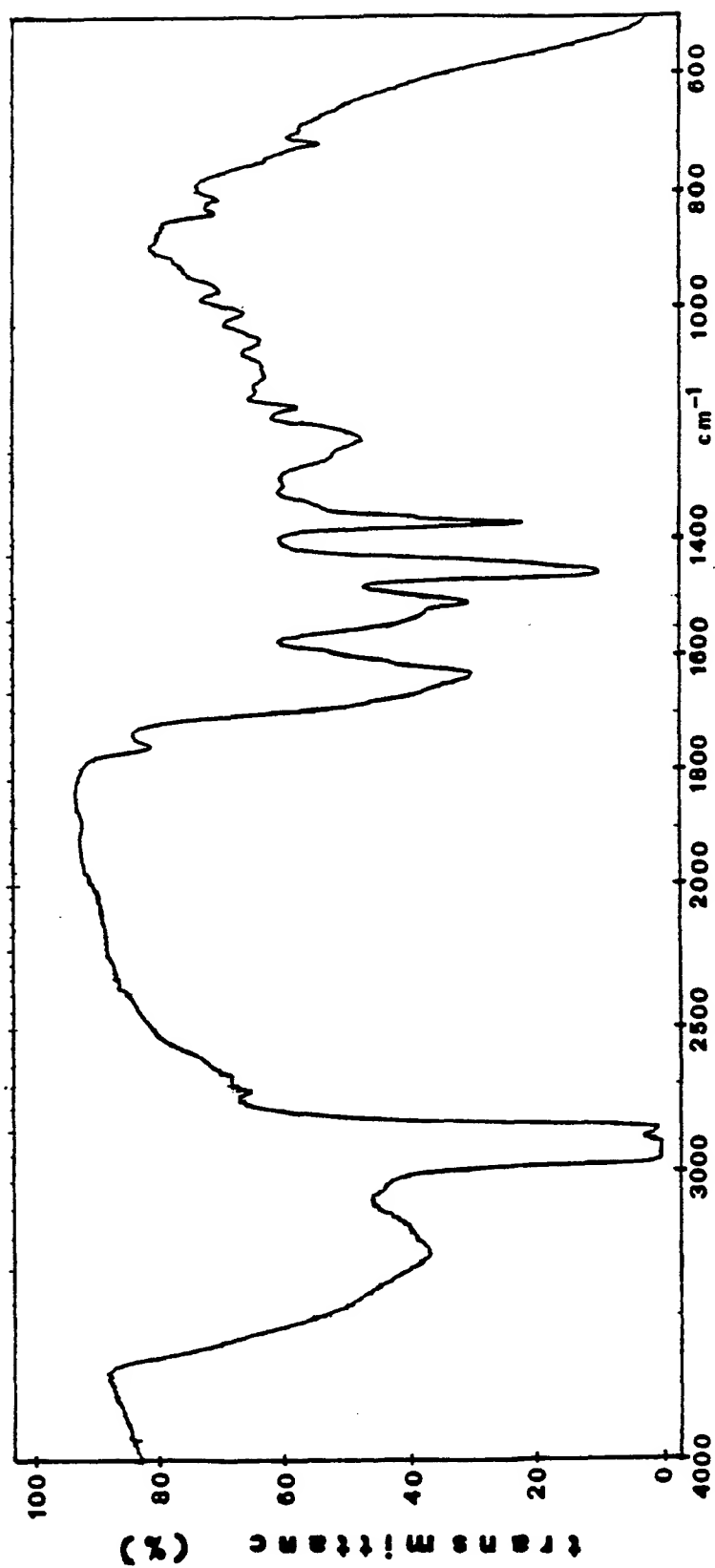


FIG. 2

ULTRAVIOLET SPECTRUM OF FACTOR A'2

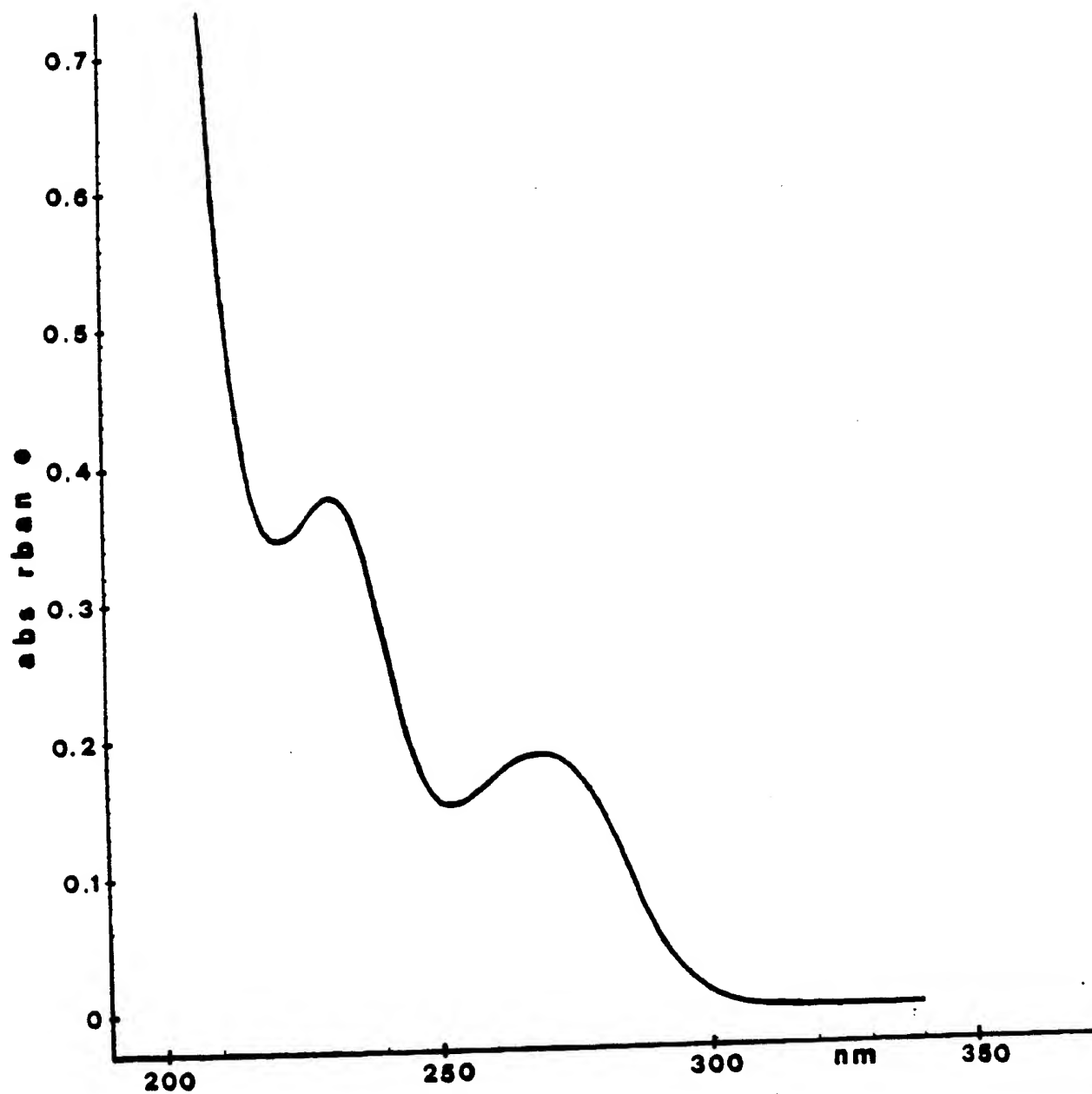


FIG. 3

①②

EUROPEAN PATENT APPLICATION

①③ Application number: **88116947.8**

①⑤ Int. Cl.⁵: **C07K 11/00, C12P 21/04,**
//A61K37/02,C07K9/00,
(C12P21/04,C12R1:045)

①④ Date of filing: **13.10.88**

③③ Priority: **27.11.87 GB 8727807**

④③ Date of publication of application:
07.06.89 Bulletin 89/23

⑧④ Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

⑧⑧ Date of deferred publication of the search report:
01.08.90 Bulletin 90/31

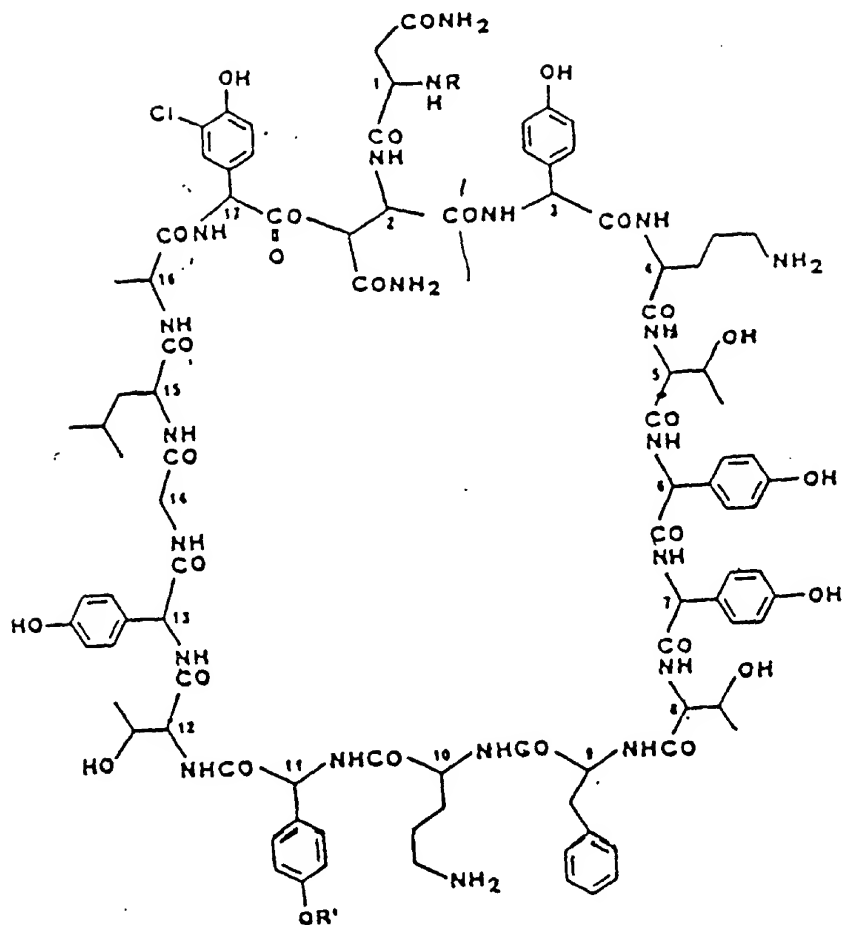
⑦① Applicant: **GRUPPO LEPETIT S.P.A.**
23, Via G. Murat
I-20159 Milano(IT)

⑦② Inventor: **Assi, Francesco**
34, Via Corridoni
I-20063 Cernusco Sul Naviglio (Milano)(IT)
Inventor: **Gastaldo, Luciano**
7, Via San Martino
I-20010 Pogliano Milanese (Milano)(IT)
Inventor: **Denaro, Maurizio**
41, Viale Bligny
I-20136 Milano(IT)
Inventor: **Ciabatti, Romeo**
15-A, Via Brodolini
I-20026 Novate Milanese (Milano)(IT)
Inventor: **Cavalleri, Bruno**
25, Via Pierlombardo
I-20135 Milano(IT)

⑤④ Novel antibiotic compounds named **A/16686 Factors A'1, A'2 and A'3.**

⑤⑦ The invention relates to depsipeptidic compounds related to antibiotic **A/16696** complex of formula I

EP 0 318 680 A3



Said compounds are named antibiotic A/16686 factors A'1, A'2 and A'3, respectively and are produced by fermentation of the microorganism *Actinoplanes* sp ATCC 33076 or a natural or artificial mutant thereof under appropriate conditions or by contacting antibiotic A/16686 factors A1, A2 and A3 or a mixture thereof with the mycelium of *Actinoplanes* sp ATCC 33076 or a natural or artificial mutant thereof. The compounds have antibacterial activity, in particular, against widely diffused gram-positive bacteria and are particularly useful for topical treating of wound infections and acne.



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 11 6947

| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
|--|--|---|---|
| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. Cl.4) |
| X | EP-A-0 046 201 (GRUPPO LEPETIT) * Claims 1-10; page 8, lines 16-26; page 9, lines 1-25; page 10, lines 12-25; page 23, lines 15-26; examples 1-4 * & US-A-4 427 656 (Cat. D) --- | 1-14,16 ,17,19 | C 07 K 11/00 C 12 P 1/04 A 61 K 37/02 // (C 07 K 9/00 C 12 P 21:04 C 12 R 1:045) |
| X | JOURNAL OF ANTIBIOTICS, vol. XXXVII, no. 4, 1984, pages 309-317, Tokyo, JP; B. CAVALLERI et al.: "A-16686, a new antibiotic from Actinoplanes; I. Fermentation, isolation and preliminary physico-chemical characteristics" * Pages 310,311,312,317 * ----- | 1-14,16 ,17,19 | |
| | | | TECHNICAL FIELDS SEARCHED (Int. Cl.4) |
| | | | C 07 K C 12 P |
| The present search report has been drawn up for all claims | | | |
| Place of search THE HAGUE | | Date of completion of the search 09-04-1990 | Examiner PEETERS J.C. |
| CATEGORY OF CITED DOCUMENTS | | | |
| X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document | | T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document | |

EPO FORM 1501 03.82 (1/0401)

4
4

